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METHODS FOR MEASURING THE COLIFORM CONTENT OF WATER

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Federal Water Pollution Control Administration
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Final Report

"METHODS FOR MEASURING THE COLIFORM CONTENT OF WATER"

PHS Grant No. WP 00202

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Final Report

"Methods for Measuring the Coliform Content of Water"

PHS Grant No. WP 00202

Principal Investigators - Joseph A. McCarthy

John E. Delaney

Date of Report - November 26, 1965

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Section 1 Improved Membrane Filter Method for Estimating the Coliform Density in Water

Introduction

A procedure has been developed under NIH Grant WP00202, which affords much greater and more uniform recovery of all the coliform types found in water samples than any combination of medium and technic previously tested. This advancement has resulted from critical studies of the behavior of the various members of the group and particularly of their growth characteristics on various media.

Proposed Procedure

a) Enrichment

The inhibiting and/or bactericidal influence shown by M-Endo broth against weak coliforms in the first hours of incubation strongly suggested the need of an enrichment or resuscitation period to provide a non-toxic environment for gradual adjustment of these weaker organisms. Experiments showed the value in providing this bridge for bacterial life between the environment of the samples and that of the M-Endo ingredients. Extensive trials on all types of samples from sewage to the best tap waters, have indicated that two hours plus or minus half an hour, is sufficient to provide adequate enrichment for the weakest coliforms, and is not stimulatory for the most rapidly growing varieties. This period appears to be used entirely for acclimating, with a minimum of logarithmic growth. The same trials showed that normal single strength lauryl tryptose broth was a very satisfactory medium.

The two-step method with enrichment thus appears to present obvious advantages in uniform recovery of all types of coliforms. The sole disadvantage, the necessity of changing the filter from the enrichment to the final selective medium is not so prohibitively time-consuming or laborious as to preclude adoption; it has been minimized by a procedure in which both phases, enrichment and final growth, are carried on in the same petri dish, with only moments required for transfer.

b) Endo Broth vs Endo Agar

Many of the disadvantages of Endo broth [1] are associated not so much with the chemical or growth character of the medium but with its physical characteristics as a substrate. The viscosity of the medium is essentially that of water, so that almost unlimited diffusion is possible. It seemed logical that if this were adjusted, the unfavorable effects of the Endo medium on the weaker organisms might well be reduced. The addition of agar-agar to the basic medium was found at once to show these advantages.

1. Enrichment, plus an agar substrate (E & A) almost without exception, produced a higher degree of coliform recovery than the Endo broth one-step technic. In some of the samples, additional recovery ran to several hundred per cent. A detailed analysis of the comparative results is presented in a later section of the report.

2. The sheen produced in colonies grown on the agar medium after enrichment was more pronounced than that on the liquid medium, and instead of fading rapidly, the sheen remained, generally without change, for many hours or even for days after its first appearance.

3. This retention of sheen made it possible and advisable to allow a full overall 24 hours of incubation, since the danger of losing the

characteristic appearance no longer exists. Colonies which develop the characteristic sheen well before the 24 hours will retain their appearance and the slower forms will be given the longer time apparently necessary. It is therefore possible to begin analysis of samples almost any time during the working day, with assurance that significant results will be available almost any time during the following day.

Formulation of Endo Agar

At first, the semi-solid medium was prepared simply by adding agar to the M-Endo broth. Subsequent investigation showed however that the fuchsin-sulfite concentration required with the agar substrate was considerably less than that needed with the liquid medium to give at least equal sheen; the relative proportions remained unchanged. Eventually it was demonstrated that the strength of the M-Endo medium could be reduced by 25% and that 1.5% of agar gave best results. The formulation of the medium which has been labeled L. E. S. Endo agar MF is as follows:

Bacto-Yeast Extract	1.2 g
Bacto-Casitone	3.7 g
Bacto-Thiopeptone	3.7 g
Bacto-Tryptose	7.5 g
Bacto-Lactose	9.4 g
Dipotassium Phosphate	3.3 g
Monopotassium Phosphate	1.0 g
Sodium Chloride	3.7 g
Sodium Desoxycholate	0.1 g
Sodium Lauryl Sulphate	0.05 g
Sodium Sulfite	1.6 g
Bacto-Basic Fuchsin	0.8 g
Agar	15.0 g

To rehydrate the medium, suspend 51 grams and 11 ml of ethyl alcohol in 1000 ml distilled water and heat to an incipient boil, agitation is advisable, cool to 45°-50°C and dispense into the smaller half of 60 cm glass petri dishes or disposable plastic dishes. A four

milliliter portion of the agar is sufficient to provide the necessary depth of medium in these two type vessels. To utilize 100 cm petri dishes, 8 to 10 ml of the agar will be necessary The agar plates should then be stored in the refrigerator and can be used over a two week period. Prolonged exposure to sunlight will cause an intensification of the color.

Procedure in Detail

In the larger half of the petri dish, an absorbent pad is placed and saturated with lauryl tryptose broth. Approximately 1.7 - 1.8 ml of broth will be sufficient. Excess liquid should be removed since it is prone to produce "sloppy" end results.

The "prepared" membrane filter is placed on the saturated pad with a rolling motion so as to prevent air entrapment. The inverted petri dish is then incubated at 35°C for a ± 1/2 hour.

After the enrichment period, the membrane is stripped from the enrichment pad and rolled onto the surface of the Endo agar in the other half of the petri dish. The enrichment incubation period has allowed the agar mass to acquire the temperature of the enrichment phase, and thus the temperature gradient at the moment of transfer is practically eliminated. Incorrect placement of the membrane on the agar is at once obvious, since patches of unstained membrane indicate the entrapment of air between the agar and membrane and the membrane must be realigned to achieve total contact. Intimate contact is not a necessity for the peripheral area outside the filtering area. The used pad may be discarded at this time or may be left in the top section for humidity purposes. The petri dish is again inverted and incubated at 35°C. After 22 hours the sheen colonies

are counted, preferably with a stereomicroscope and with a light source above and approximately perpendicular to the plane of the membrane. Other colonies may be counted, frequently affording valuable information.

Comparison of Standard Multitube Procedure and Membrane Filter E&A Method

Of the routine drinking water samples received at the Lawrence Experiment Station during a two month period, 654 were examined in parallel by the standard lactose broth multitube procedure and by the new E & A technic. The samples came from the Massachusetts municipal supplies, from Army and Navy facilities, and from State recreational areas. The results are shown in Table I.

Table I

Comparison of Standard Procedure and E & A Membrane Filter

	E & A Membrane Filter			Standard Fermentation Tube		
Total Number	Samples with no sheen colonies	Samples with one or more sheen colonies	False Positives	Samples with no Positive Presumptives	Samples with no Confirmed Positives	Samples with Completed Positives
654	567	87	3	389	220	45

For each sample, a volume of 100 ml was taken for examination by MF, and the usual five portions of 10 ml each were inoculated into the standard broth tubes.

The data indicate that 87% of all the samples were found free of coliform by the MF technic; all of these could be reported as negative one day after the receipt of the sample. By the fermentation tube technic, because of the requirement for 48 hours incubation, only 59.5% of the samples

could be reported as negative after two days. After another 48 hours, the time required for reporting a negative result in the confirmed test on presumptive positives, another 33.7% of the samples could be reported as negative.

The E & A MF technic showed one or more verified coliform organisms from 84 of the samples, while only 45 samples had one or more positive completed tubes from broth. In four samples, positive results were found by the completed test on one tube each, and no coliform were reported from the MF test on these samples. There were 43 samples from which verified MF coliforms were found, in which no coliforms were isolated by the standard procedure. Some of this disagreement might be attributed to the larger volume of sample examined by the MF test.

On the 87 samples from which positive MF tests were found, only three were shown in which all the sheen colonies failed to verify. By comparison, of the 3270 total tubes inoculated from the samples, 522 positive lactose tubes failed to confirm; in other words about 16% of all the tubes inoculated were processed through the confirmation test without any positive coliform results. This is a rather important picture of the excess work which the standard fermentation tube method may involve.

The data assembled in Table I definitely indicate that under the conditions of this comparison, the E & A MF technic operated a higher recovery efficiency than did the Standard Lactose Broth test. Even if the effect of the larger volume is discounted, it appears that the E & A technic finds larger numbers of coliforms than the Standard Procedure in waters of drinking quality. Many observers have from time to time reported that the Standard Procedure recovers only a part of all the coliforms present. For example, much larger coliform recovery has been reported from the droplet method.

E & A Membrane Filter Method Applied to Polluted Waters

The results obtained by the E & A membrane filter method on waters not of drinking water quality are reported in graphical form in Figures I, II, and III.

Figure I represents the analyses of 49 river, pond and lake samples that were examined by the E & A, one step M-Endo broth and the 5-5-5 multitube technics. The ratios of the MPN to E & A and the MPN to M-Endo broth (one step) results were calculated and arrayed in order of magnitude. The two sets of data were then plotted on log probability paper. The two lines of best fit show the difference in response produced by the two membrane filter methods.

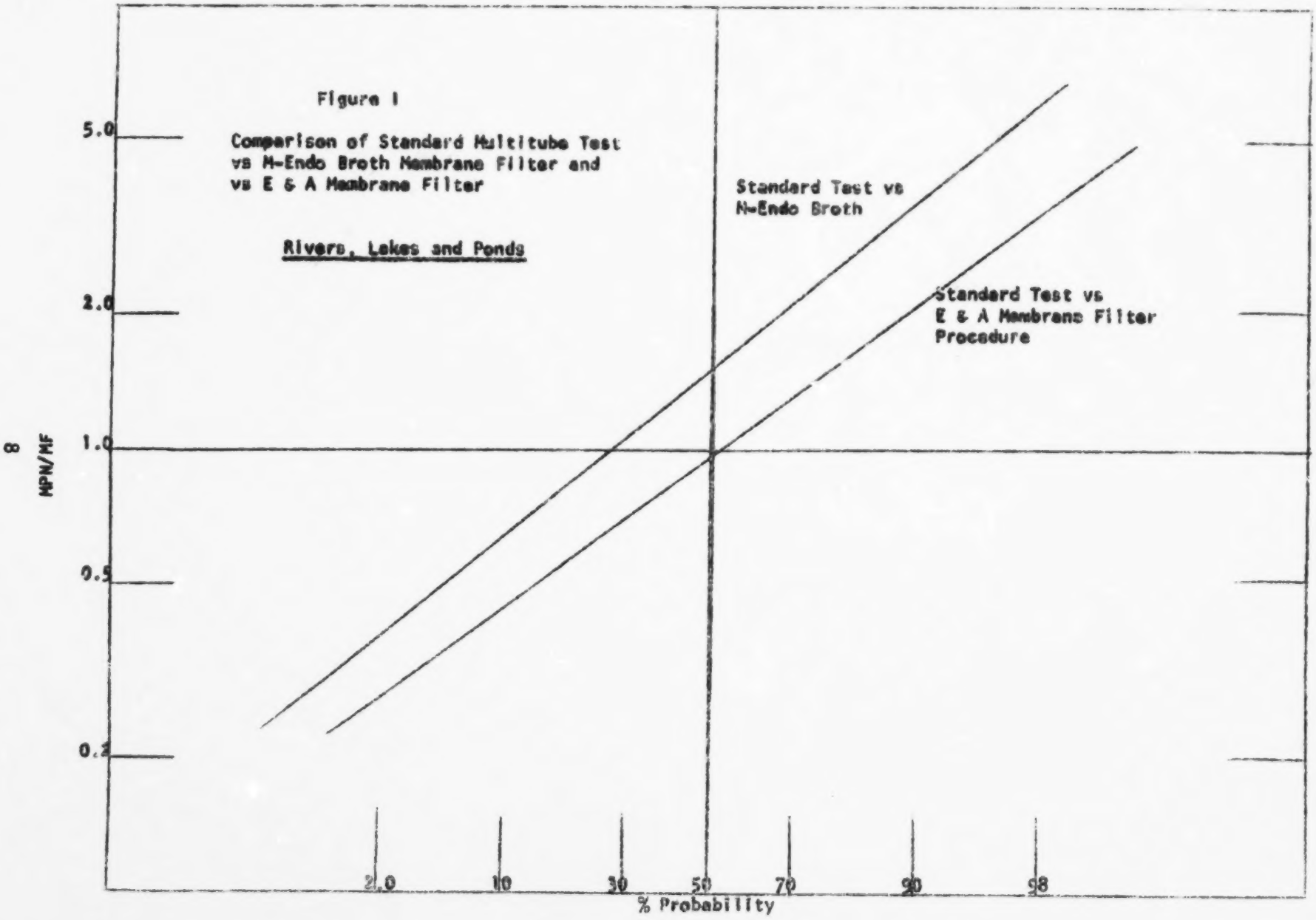
Of the 49 ratios $(\frac{MPN}{MF})$ calculated from the one-step membrane filter method, 39 were equal or greater than unity, while the MPN/E & A array showed that 23 ratios were equal or greater than unity. This comparison shows that the one-step membrane filter method using M-Endo broth as usual tends to give lower results than the MPN procedure, while the E & A membrane filter analysis of waters of these types gives results practically equal to those from the tube method. The median ratio or 50 percentile ratio for the MPN/one step MF is 1.53 while the MPN/E&A MF is 0.97.

Figure III represents the analyses of 49 raw, settled and biologically treated sewage samples, by the 5-5-5 multitube method and by the E & A membrane filter method. The ratios of MPN to E & A coliform densities were calculated, arrayed and plotted on log probability paper. The median or 50 percentile ratio of the 49 ratios is 1.10, with 26 ratios equal or greater than unity.

Figure 1

Comparison of Standard Multitube Test vs M-Endo Broth Membrane Filter and vs E & A Membrane Filter

Rivers, Lakes and Ponds



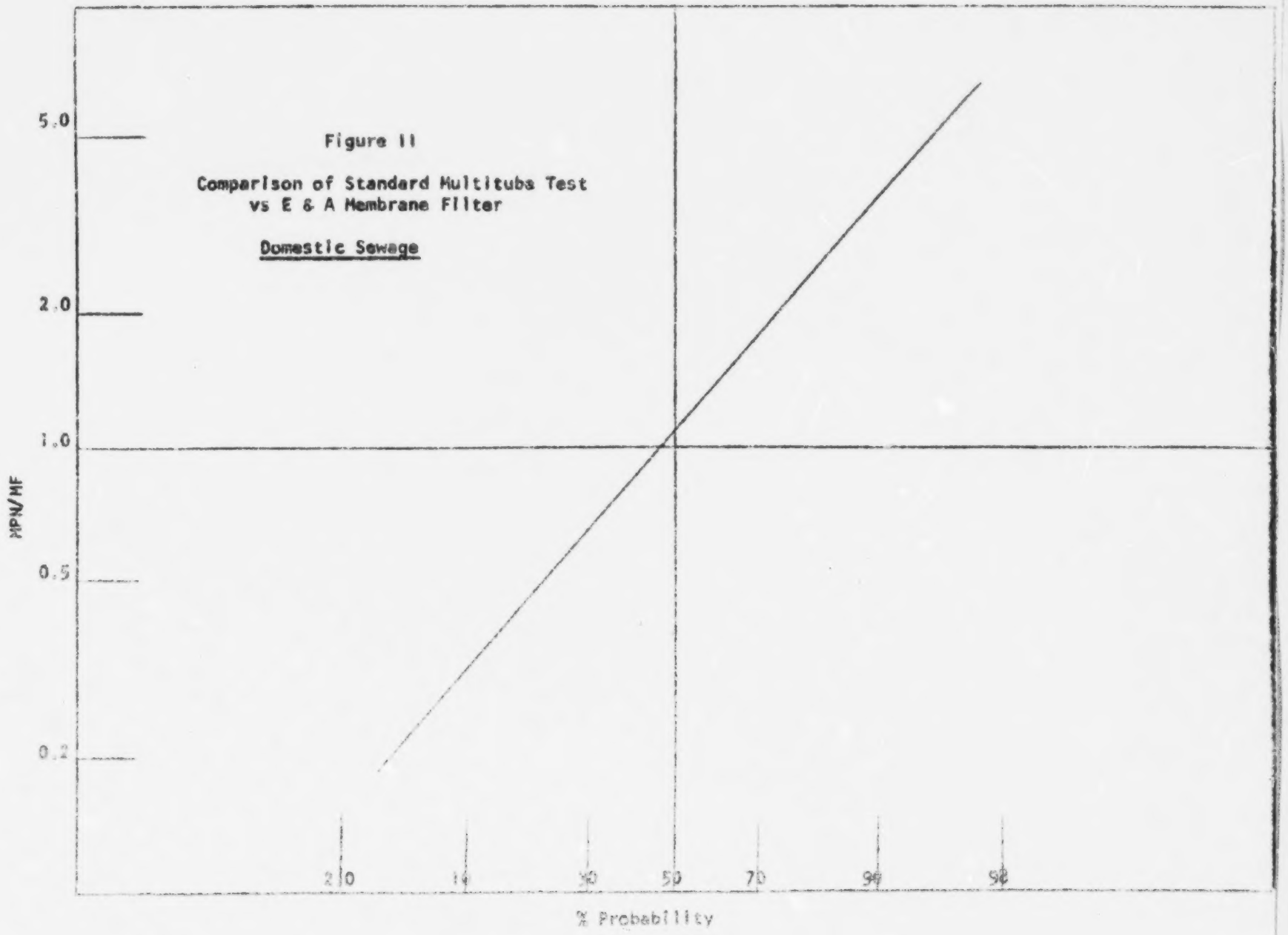


Figure 11

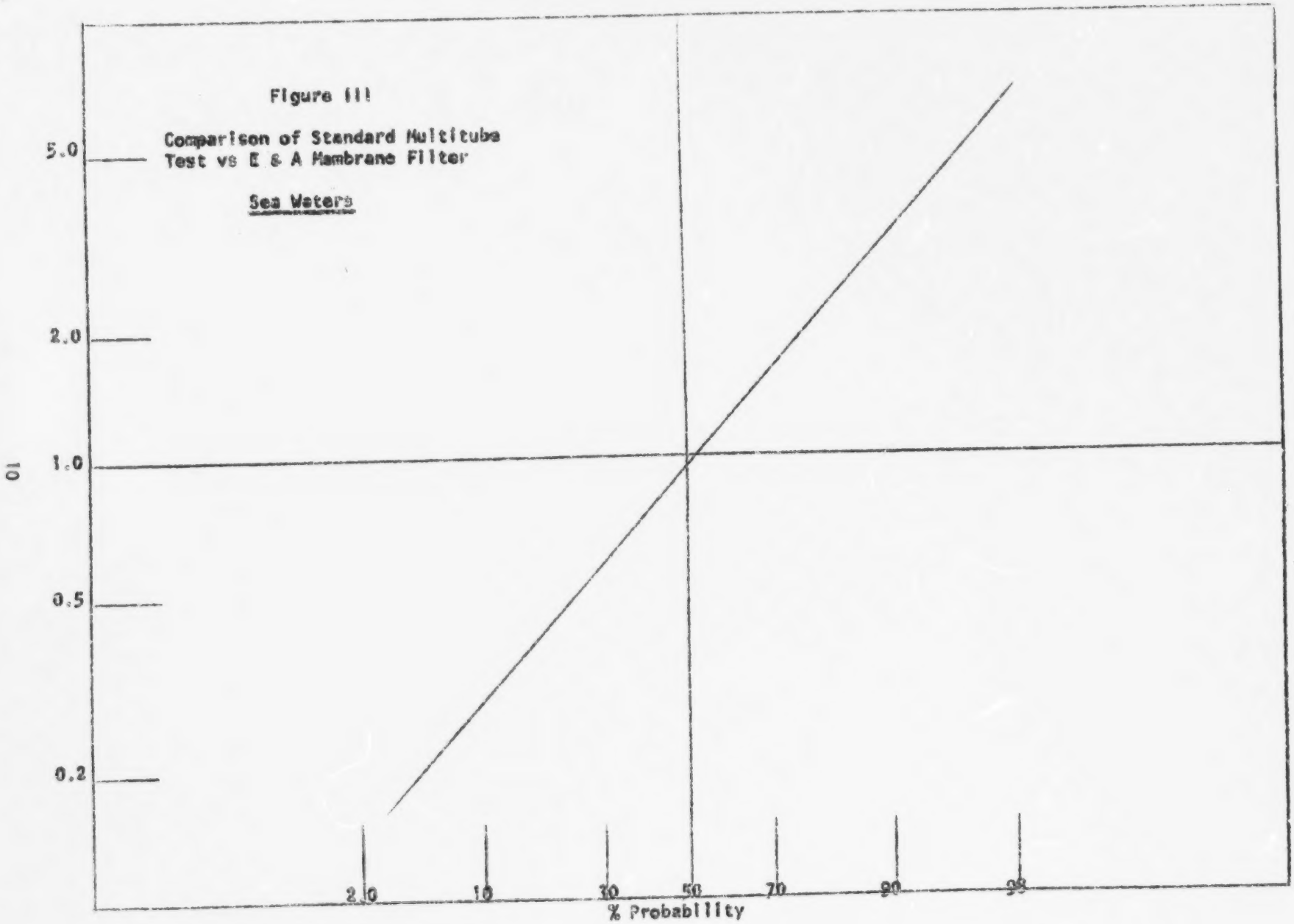
Comparison of Standard Multitube Test
vs E & A Membrane Filter

Domestic Sewage

Figure III

Comparison of Standard Multitube Test vs E & A Membrane Filter

Sea Waters



An independent comparison of the coliform recovery efficiency of the E & A technic and a one-step agar method indicated that the enrichment phase did not increase materially the coliforms recovered from sewages and polluted rivers. These results confirmed well known beliefs that the nearer the source of pollution, the less attenuated are the coliform strains, and therefore the smaller the numbers of coliforms which fail to withstand the inhibitory effects of the environment of the various bacteriological procedures. In this study this appears to be particularly true of the inhibitions observed in the fuchsin-sulfite atmosphere of the MF test.

It probably therefore will be possible to recommend that on examinations of sewages and highly polluted rivers and streams the enrichment stage of the E & A membrane technic may be omitted without significant effect on the coliform recovery. It is very probable that considerable reduction in the non-coliform background will also be found.

Figure III presents the array of 49 3-3-3 multitube and E & A membrane filter ratios calculated from simultaneous analyses on sea waters. The filters were washed with sterile buffered water in the same manner as that used in drinking waters. It was not found necessary to use three to five additional washes, as had been earlier indicated. The 50 percentile of the array is 0.96 and 24 of the MPN/MF ratios were equal or greater than unity, thus indicating a very close agreement with the tube MPN.

Summary

In our paper on the reliability of coliform tests [2] we stated the membrane filter appeared to possess the best possibility of a test with greater precision than the tube MPN and enumeration of all strains of coliform. The results presented in this report indicate that a considerable step has been taken toward these goals.

1. McCarthy, J. A., Delaney, J. E., Grasso, R. J. Measuring Coliform In Water Water & Sewage Works, June 1961

2. McCarthy, J. A., Thomas, H. A., Jr., Delaney, J. E. Evaluation of the Reliability of Coliform Density Tests AJPH 48: 1628 (1958)

Section II Measurement of E. coli type I by the Membrane Filter

Introduction

Since the isolation of B. coli from the feces of a cholera patient [1] by Escherich in 1885 and the subsequent finding that it occurred regularly and in enormous numbers in the excreta of man and many other animals, there has been a continuing attempt to develop an accurate, specific and relatively simple test for estimating the density of this organism in water.

Multitube Test for Estimating E. coli Type I Densities

The procedure currently used for determining the density of E. coli Type I in water has been the multitube technic using a presumptive lactose fermentation at 35°C. followed by a confirmation fermentation in the E. C. Medium of Hajna and Perry at 44.5°C. This method per se is saddled with the inherent disadvantages of all MPN procedures, namely, low precision, generally low accuracy and lengthy time of analysis, plus two disadvantages specific to this selective procedure. These are: (1) the necessity of having two incubators to satisfy the temperature requirements of the presumptive and confirmed tests and (2) the ability of other coliform strains to produce a positive EC confirmed reaction. Other investigators have shown that 44.5°C. positive lactose fermenting strains exist within most IMViC biotypes and that consistently the irregular type VI (- - + +) and irregular type II (- + - -) constitute the strains that interfere most often with the specificity of the EC test. The relative incidence of these interfering strains varies from water to water, with a resulting fluctuation in error.

Membrane Filter for Estimating E. coli Type I Densities

The low specificity, the inherent poor precision and the prolonged time of analysis required for the EC multitube procedure, together with the ability to use larger volumes of sample with the membrane filter procedure, stimulated our interest in determining whether the membrane filter could be used for estimating E. coli densities in waters. The important question was the degree of accuracy that could be incorporated into an E. coli membrane filter technic.

It is entirely feasible to obtain accurate E. coli Type I density measurements by using the E & A coliform membrane filter method and then differentiating all the sheen colonies. This procedure, however, is time consuming and obviously precludes routine use.

At the outset, it seemed compulsory to incorporate a high incubation temperature into our procedure as one aid to ensure bacterial separation. The difficulty of obtaining a fuchsin-sulfite concentration that would be stable and yield sheen at an elevated temperature, and the already demonstrated knowledge that strains of coliform other than E. coli as shown in Table I could grow and ferment lactose at the higher temperature, eliminated for consideration any Endo modification and any lactose based medium.

The common biochemical reactions, better known as the IMViC tests, were then reviewed for a possible means of bacterial separation and identification. The ability of E. coli Type I to produce indol from tryptophan at an elevated temperature [2, 3] indicated a possible basis for a selective test, especially since the two coliform strains most likely to interfere with the broth tests at an elevated temperature, namely Irregular VI (- - + +)

and Irregular II (- + - -) are fortunately indol negative and thus would be automatically eliminated from interfering with a test based on indol production.

Preliminary conception of the procedure centered on growing the E. coli Type I organisms on a tryptone (a peptone particularly rich in tryptophan) agar at an elevated temperature and then staining the colonies to determine the indol formers.

Procedure

(a) Growth of E. coli Type I

After the membrane filters are prepared in the usual manner they are placed on a Tryptone Bile Agar (TBA) of the following composition:

Tryptone	20 gms
Bile Salt No. 3	1.5 gms
Agar Agar	15 gms
Distilled Water	1000 ml
pH	7.0 - 7.2

Four ml of the melted agar are pipetted into a plastic petri dish 49 mm in diameter and provided with a tight cover to prevent excessive dehydration during the incubation period. The filters are incubated at 44.5°C. for 20-24 hr. This temperature is extremely critical. A lower incubation temperature will permit false reactions to occur and a higher temperature will be suppressive to several E. coli Type I strains.

(b) Staining Procedure

Two solutions are necessary to give the best color development of indol positive colonies.

Solution A - 2.5 gms of para dimethylaminobenzaldehyde
are dissolved in 90 ml of ethyl alcohol and
10 ml of concentrated HCl added to the solution.

Solution B - 3.5% solution of Oxone ($KHSO_5$)

Separation of the two solutions is necessary to avoid the oxidation effects of oxone.

The staining technic consists of first mixing 1 ml of Solution A and 1 ml of Solution B in one half of a 60 mm glass petri dish and then placing a regular membrane filter absorbent pad in the mixture. The incubated membrane is stripped from the TBA and placed on the saturated pad. Color development will be observed on the indol forming colonies in less than thirty seconds. It was found that heating greatly intensified the color. Localized heating of the membrane surface was accomplished by subjecting the filter to a two minute exposure of infra red light from a distance of 4 to 6 in.

(c) Counting of Indol Positive Colonies

After exposure to the heat, the positive colonies are very easily distinguished. They are deep red and as a rule they are from 2-4 mm in diameter. The indol negative colonies stain yellow to brown.

If further processing of the TBA colonies is desired, it will be necessary to "fish" them prior to the staining procedure due to its bactericidal action.

Accuracy of the TBA Procedure for E. coli Type I

It has been shown in our report [4] on the E & A technic that this procedure is very accurate for the recovery of coliform organisms and therefore it follows that it is as accurate for E. coli Type I organisms. Because of the

poor confidence and overestimation associated with the EC MPN test it was decided to use the E & A technic to check the accuracy of the proposed TBA procedure for E. coli Type I. The obvious procedure was to differentiate all the discrete sheen colonies developed by the E & A method and compare the number of E. coli thus obtained with the number of red colonies developed by the TBA procedure. This technic pits a direct count procedure against a direct count procedure, thus eliminating all bias, low confidence, poor reproducibility, etc. that would plague a comparative study between a "probable" and direct count.

Table I presents the data collected from eight samples of Haggetts Pond, a surface water with a coliform MPN normally ranging between 10 to 50. Twenty membranes were prepared from each sample and ten were processed by the E & A technic and ten by the TBA method. All the sheen colonies on the E & A membranes and all the colonies appearing on the TBA membranes were verified for coliform characteristics and differentiated. The TBA colonies were fished prior to staining. The results were then sorted into groups. The numbers of E. coli Type I recovered by the two technics are in very good agreement. The actual total recovery from the eight separate samples numbered 183 by the E & A technic and 168 by the TBA method. When the recovery is compared to the "red" colonies developed on the TBA membranes after staining, as would be the case in practice, the comparison is even better, 183 to 180. On the basis of individual samples, the ratios between E. coli Type I organisms grown by the E & A technic and the "red" colonies developed by the TBA technic range from 0.6 to 1.29 with a median of .91 for an overall ratio of 1.02.

Note: Similar studies made while using polluted waters gave excellent recovery values for E. coli type I by the TBA method. [5]

Table 1

E. coli T[illegible] I Recovered by (1) E & A and (2) TBA Technic

Sample-Haggetts Pond

Coliform Biotypes	Sample #1 (1)	Sample #1 (2)	Sample #2 (1)	Sample #2 (2)	Sample #3 (1)	Sample #3 (2)	Sample #4 (1)	Sample #4 (2)	Sample #5 (1)	Sample #5 (2)	Sample #6 (1)	Sample #6 (2)	Sample #7 (1)	Sample #7 (2)	Sample #8 (1)	Sample #8 (2)	Totals (1)	Totals (2)
+ + - -	15	12	75	58	6	9	24	32	19	19	24	17	8	9	12	12	183	168
- - + +	10	0	10	2	4	2	16	0	9	5	12	0	1	1	12	0	74	9
- + - -	0	0	5	0	0	0	0	2	0	0	3	0	1	0	0	0	9	2
- + - +	20	0	125	1	6	0	24	0	8	0	7	0	2	0	7	0	199	1
+ + - +	0	0	10	0	0	0	0	0	6	0	1	0	0	0	0	0	17	0
+ - + +	0	0	5	0	0	0	0	0	4	0	11	0	1	0	3	1	25	1
- + + +	5	0	20	1	1	0	4	0	1	0	2	0	0	0	0	0	33	1
- - + -	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0
Red Colonies		12		58		10		34		23		21		9		13		180
Ratio $\frac{++--\text{by(1)}}{\text{Reds}}$	1.25		1.29		0.6		.71		.83		1.14		.89		.92		1.02	

Table II presents the data on the selectivity of the TBA method achieved on twenty samples of water of varying pollution. For presentation purposes the samples have been grouped into three categories, namely, sewages, polluted rivers and pond waters. The reliability that a red colony developed on the TBA membrane is actually an E. coli Type I appears to be good to excellent, with individual sample verification ranging from 81 to 100 percent with a median of 92.8 percent. For comparison purposes, data for the coliform selectivity of the E & A test for the same samples are also presented. For individual samples, this verification ranges from 69 to 98 percent with a median of 89.3 percent. A comparison of the total number of colonies examined by both methods shows that out of 581 red colonies, 529 or 91.4 percent were typed out as E. coli Type I organisms while out of 1860 sheen colonies, 1658 or 89 percent were shown to be fostered by a coliform organism. The TBA method, therefore, showed false positives in only 8.6 percent of the cultures tested while 11 percent of the sheen colonies fostered by the E & A method failed to meet the definition of the coliform group.

This table also shows the percentages of the total E & A growth able to survive and grow under the TBA conditions. Generally, the more polluted the water sample the greater the percentage of survivors, with sewage showing between 10-20 percent, polluted rivers 2-10 percent and pond water 1-2 percent. This trait is naturally advantageous since it permits a larger sample to be analyzed and avoids the effects of interfering background growth.

Summary

A direct count method for E. coli Type I in water is presented and is based on a new medial concept for the quantification of this

Table II

Selectivity of the TBA Method for E. coli Type I vs Selectivity of E & A Method for Coliform

TBA Selectivity

Type Sample	Number	E&A Growth on TBA	Red Colonies	Verified	%	Range of Individual Samples (%)
Sewage	6	10 to 20%	263	234	89.8	83 to 100
Polluted Rivers	6	1 to 10%	133	125	94.0	85 to 100
Pond Water	8	1 to 2%	185	68	91.0	81 to 100
Total	20		581	529	91.4	

E & A Selectivity

Type Sample	Number	Sheen Colonies	Verified	%	Range of Individual Sample (%)
Sewages	6	661	607	92.0	85 to 98
Polluted Rivers	6	828	738	89.	85 to 97
Pond Water	8	371	313	86.8	69 to 93
Total	20	1860	1658	89.	

organism. The classical use of a lactose based medium was abandoned in preference for one with a tryptone base. The specificity of the method centers on (1) the ability of this organism to transform tryptophan into indol when incubated at an elevated temperature and (2) the fortunate existence of only a few additional bacterial species that can accomplish this reaction under the prescribed incubation environment.

The TBA method has been shown to possess high levels of selectiveness and inclusiveness and therefore, of accuracy. The multitube test, however, while using a presumptive fermentation at 35°C. followed by a confirmatory fermentation at 44.5°C. has been shown to possess good inclusiveness but only fair selectiveness. The TBA method reduces the testing period for E. coli Type I enmeration to 24 hours as opposed to the 96 hours necessary for the multitube result. In fact, a bacteriologist, in order to achieve an estimation of E. coli Type I in a water with the same degree of accuracy as produced by the TBA method, must use either the one day E & A procedure followed by the 4 day verification and differential procedures for a total of 5 days or the four day EC MPN procedure followed by the 5 day isolation and differential test for a total elapsed time of nine days. The new TBA method requires only one incubation temperature.

Section III - Delayed Holding Procedure for Coliform Bacteria

Introduction

Ever since the first bacterial analyses of water were made, there has been a question regarding the possible effects of the time which has elapsed since the sample was taken. Thus a shadow has been cast on the reliability of the results of every sample, especially after any considerable period of storage, and the trueness of the results, regardless of the accuracy of the technic of the examination. Many investigators have studied this problem, but it has not been possible, with any degree of agreement, to assess the effects of the storage on the true coliform density of the water. There are so many factors which might increase biological growth or on the other hand produce a dieaway, that any clear-cut answer to the question, especially one that would apply to all kinds of water, seems almost unattainable. McCarthy [1] and Geldreich [2] have shown that, in general, samples containing a high coliform density tend to show considerable reduction while water relatively low in coliforms shows more stability, at least over a one or two day period.

The inherent difficulties of the problem can obviously be attributed to the myriad factors which affect either growth or death of the sensitive bacteria. The difficulty of prophesying these factors is all the more obvious when one considers the wide range of variety in time, distance and other conditions.

Ideally, of course, bacteriological examinations of water samples should be initiated immediately after collection; this desirable procedure is hardly ever possible. In several editions of Standard Methods, limits

were set up for duration of shipping, as well as explicit instructions in regard to acceptable temperature conditions. The 11th Edition recognized that few or even none of these precepts were being followed, and therefore made the simple recommendation that "technical procedures should be started preferably within one hour after collection and in no case should exceed 24 hours" [3] However, it is not always possible for samples to reach the laboratory within 24 hours, especially these laboratories which serve wide areas.

For these reasons, many attempts have been made to adapt or develop methods by which analyses could be made in the field, or begun at least in the field and sent to the laboratory for completion.

Application of Membrane Filter Technic to Problem

During the development of the membrane filter and its general acceptance as a tool for estimation of coliform content, it was also recognized as offering a potential solution to the problem of coliform dieaway or multiplication experienced haphazardly in a bottled sample during a storage period. Basically, the most attractive characteristic of the membrane filter which promised usefulness for reducing the problems in shipping is its ability to sieve bacteria from the liquid phase and to isolate them on a solid surface. This would prevent the normal binary fission of bacteria in the sample in liquid form in transit, which could produce more colonies than the true number, since each colony eventually counted is generally attributed to growth from a single organism.

On the other hand, dieaway in transit is visualized as a result of lack of essential food, and of antagonistic materials which are often

present in a sample stored in liquid form, and may even develop in transit. The filter would eliminate these two all-important factors. It also offers other substantial advantages which justified further investigation of the membrane filter as a transport vehicle such as (1) decrease in the weight of the sample, with a resulting lowering of the cost of shipping; (2) elimination of the necessity for refrigeration; (3) reduction of the potential breaking of glassware and the spilling of liquid; (4) a reduction of the importance of the time elapsed between collection and arrival at the laboratory; (5) and perhaps even more important, all the advantages inherent in the use of a direct count.

The MF holding technic as visualized by early investigators included the following steps: (1) filtration of the sample in the field; (2) placement of the filter on a holding medium; (3) on receipt at the laboratory, transfer of the filter to a growth medium for the necessary added time for growth; and (4) final counting.

Preliminary Consideration of the Problem

Although both the practical and the theoretical advantages of this use of the membrane filter as the vehicle of transport appeared sound and attractive, the actual application for the purpose necessitated the solution of many interlaced and exacting problems of nutrition, timing, and inhibition.

At the start of the study, it was obvious that a critical balance between nutrients and inhibitors would have to be incorporated into a holding medium so that the technic would be valid over considerable ranges of time and temperature. This exacting requirement becomes more obvious and pronounced when one considers the variety of strains which

comprise the coliform group, and the differences which exist among the strains in regard to optimum conditions of time, temperature and variation of response toward inhibitory agents and conditions.

Theoretically, the ideal medium would permit the transport of coliforms on the membrane surface with neither multiplication or dieaway during the shipping period and no carryover of growth-suppressive agents at the time of transfer to the final growth medium. Such an ideal medium is probably not to be expected. The maximum attainment would seem to be the formulation of a medium which would sustain bacterial life with a minimum of multiplication, largely prevent overgrowth, and permit the optimum growth of characteristic sheen during the final phase; and retaining these effects over fairly extended periods of time, especially during periods of high temperature. The closer the procedure approaches the ideal of "sustaining life without multiplication" the better would be the chances of obtaining results equivalent to those obtained by the usual laboratory filter technic.

After considerable preliminary experimentation, [4] a formula was adopted which showed exceptional promise. Subsequent testing of this formula with a variety of waters and a wide range of environmental conditions gave a very satisfactory response. The only difficulty which deterred us from a full-scale test of the procedure was the occasional appearance of mold growth which made counting difficult because of spreading and overgrowth. After several antifungal materials were tried, it was found that a relatively new crystalline antibiotic, Acti-dione [5] showed great specificity against molds and yeasts, and could be incorporated into the medium without any bacteriocidal or bacteriostatic effects. The formulation eventually chosen is shown below.

LES MF HOLDING MEDIUM - COLIFORM

Tryptone	3.0 grams
M-Endo Broth MF	3.0 grams
Dipotassium Hydrogen Phosphate	3.0 grams
Sodium Benzoate	1.0 grams
Sulfanilamide	1.0 grams
Para Aminobenzoic Acid	1.2 grams
Acti-dione	0.5 grams
Water	1000 ml

Preparation: Dissolve 12.7 grams in 1 liter of distilled water. No heating is necessary. Final pH 7.1 $\pm$ 0.1.

Materials: Culture dishes: Sterile plastic petri dishes (50 x 12 mm) disposable and moisture tight are recommended. Such containers are low in weight and do not break in transit.

Procedure: 1. Filtration: The membrane filter is prepared in the same fashion as when used for immediate incubation.

2. Sample Preservation and Shipment: Place absorbent pad into petri dish and saturate with LES MF Coliform Holding Medium. Two milliliters are sufficient to saturate most absorbent pads. Permit the pad and medium to stand for a few minutes before pouring off excess. Remove the membrane filter from the filtration unit with sterile forceps, and roll it, grid side up, onto the surface of the saturated pad. The humidity is preserved by the tight closure afforded by the plastic petri dish. The culture dish is then transferred to a shipping container and sent to the laboratory for completion of the examination procedure.

The sender must use discretion at this step of the procedure, in order to assure that during the first hours of storage, the membrane is not exposed to extreme cold or excessively hot temperature conditions.

3. Incubation: At the laboratory, the membrane filter is transferred from the holding environment to LES Endo Agar (growth and indicator medium). At the time of transfer, if the bacteriologist observes distinct colony formation on the membrane surface without the aid of magnification, it is recommended that the transfer be made and the petri dish then refrigerated until late in the day when it is incubated at 35°C for a 16 hour period. All other membranes after transfer are incubated for 20 hours. This manipulation of the incubation time will permit the bacteriologist to exert a measure of control on the problems of overgrowth and the dissipation of sheen that interfere with coliform colony count.

4. Counting: All dark colonies having a metallic-appearing surface luster are counted as coliform colonies. The counts are made with the aid of a low power microscope or other suitable optical device, using a light source above and approximately perpendicular to the plane of the filter membrane.

Before the new LES Holding Technic could be considered fully acceptable, it was necessary to apply the method to a long term study using natural waters under variable conditions of temperature and storage. After lengthy appraisal and discussion of the importance of the variables which would affect the technic, it was decided to compare the recoveries of the Tentative Standard Method against the LES Method (1) over a period of 48 hours; (2) during a range of temperature consistent with the ambient temperature at the time of collection, and (3) on a variety of surface waters. The 48 hour period was selected because it was felt that the very great majority of samples would reach the laboratory within this time, and that all coliform species which can normally be cultivated in 48 hours could be expected to be recoverable at intervals prior to 48 hours storage. The study could have included 72 and 96 hour storage, but it seemed that less valuable information could have been obtained in this way than in testing the much larger number of samples which could have been examined with a 48 hour period alone.

The temperature conditions chosen for the study were based on the reasoning that samples in transit would very likely be exposed to fluctuating temperatures. It was also felt that the temperature of the water at the point of collection had considerable effects on the types and viability of the coliforms present, and that any fixed incubation cycle, say at 5°C during the summer, or 35°C in the winter would have imposed unrealistic pressures on the bacterial community filtered onto the membrane filter during these seasons. Finally, a varied program of samples was chosen to allow the evaluation of the new procedure not only

on one type of water with perhaps fecal types predominant, but on several sources in which could be found a good representation of all coliform strains in various stages of attenuation.

As soon as possible after collection, fifteen membranes were prepared and five of each were randomly placed on (1) LES Endo Agar, (2) LES Holding Broth, and (3) Standard Method Holding Broth. Five replicate MPN determinations were set up at the same time. The membranes on the LES Endo Agar and the MPN tubes were incubated at 35°C and the usual steps in determination of coliform were carried on in subsequent days. The Holding membranes and the remainder of the original water sample were held for 48 hours at a temperature consistent with the season of the year. At the termination of the holding period the membranes on the holding media were transferred to the appropriate growth medium, that is the membranes on LES Holding Medium were transferred to LES Endo Agar and those on the Standard Method medium were transferred to M-Endo Broth. The transferred membranes were then incubated for the required time at 35°C, and the coliform count was made. The stored water sample was analyzed for its coliform concentration by five replicate MPN determinations and by five replicate membrane filter analyses. All MPN tests were carried through confirmation.

After the tests were completed, the replicate membrane counts were averaged arithmetically and the MPN estimates were averaged logarithmetically. The results from 50 samples collected during the months of December, January and February and the results from 50 samples collected during the months of June, July and August were averaged in this manner. In order to present the data in perhaps a more informative manner, and to provide an answer for

the variety of results, the important ratios of each set of samples were calculated, arrayed in order of magnitude, and plotted on log-probability paper as shown in Fig. I and Fig. II respectively.

Discussion

The data consolidated in probability plots of Figure I and Figure II indicate quite conclusively the higher coliform recovery potential of the LES Holding Technic over that of either the Standard Methods Holding Procedure or the stored water environment.

Table I shows the median ratio and the 25-75 percentile range of the ratios obtained during the cold weather study while Table II shows these statistics for the ratios obtained during the summer months. Fifty samples were analyzed during each period.

Table I

(sample collection - December, January, February)

Ratio	Median Ratio	25-75 Percentile Range
LES HOLDING COUNT / Initial MF Count	1.06	0.94 - 1.25
S.M. HOLDING COUNT / Initial MF Count	0.59	0.47 - 0.80
STORED WATER MF COUNT / Initial MF Count	0.71	0.25 - 1.40

Table II

(sample collection - June, July, August)

Ratio	Median Ratio	25-75 Percentile Range
LES HOLDING COUNT / Initial MF Count	1.02	0.88 - 1.18
S.M. HOLDING COUNT / Initial MF Count	0.81	0.62 - 1.07
STORED WATER MPN / Initial MPN	0.51	0.29 - 0.90

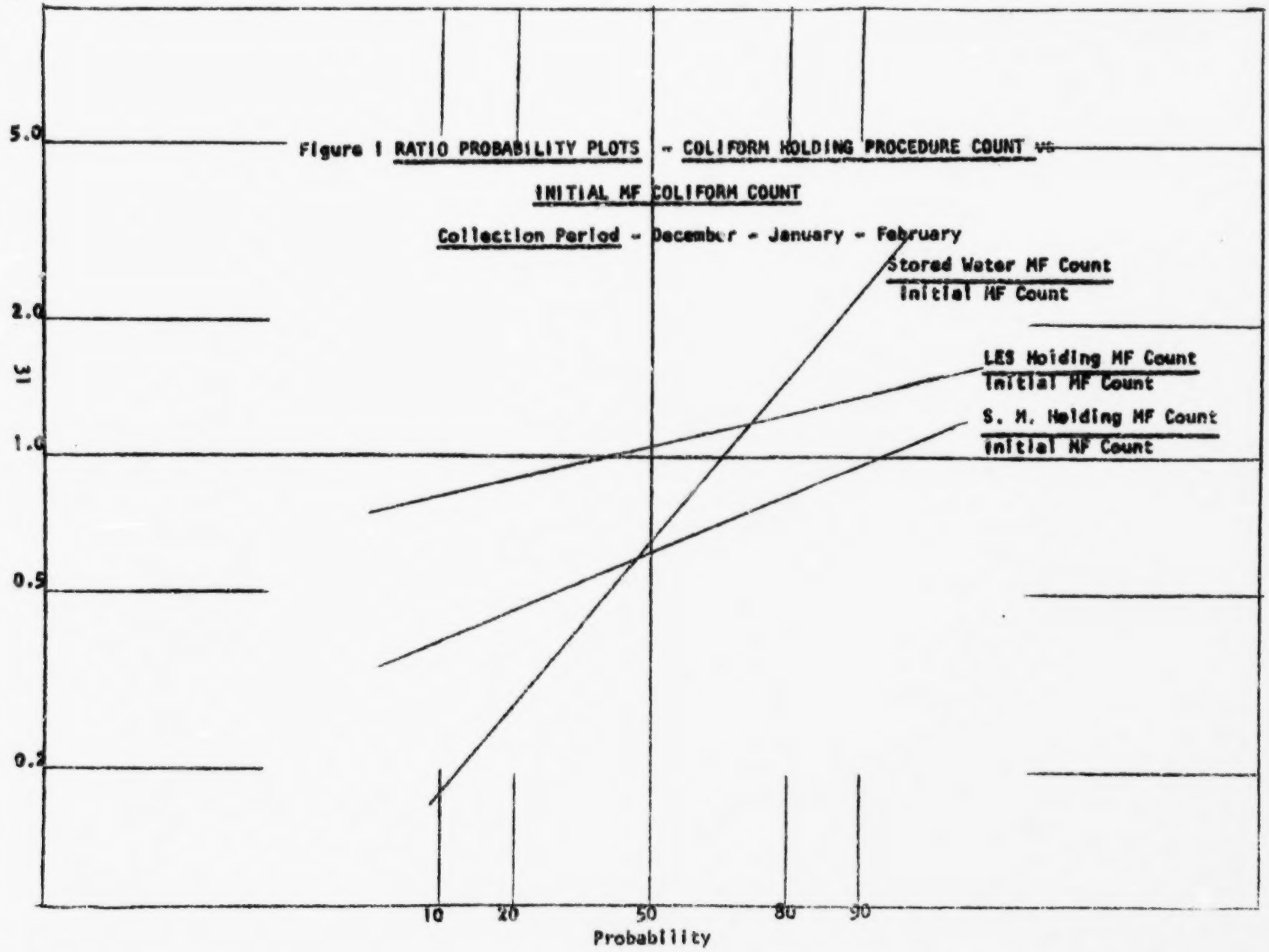
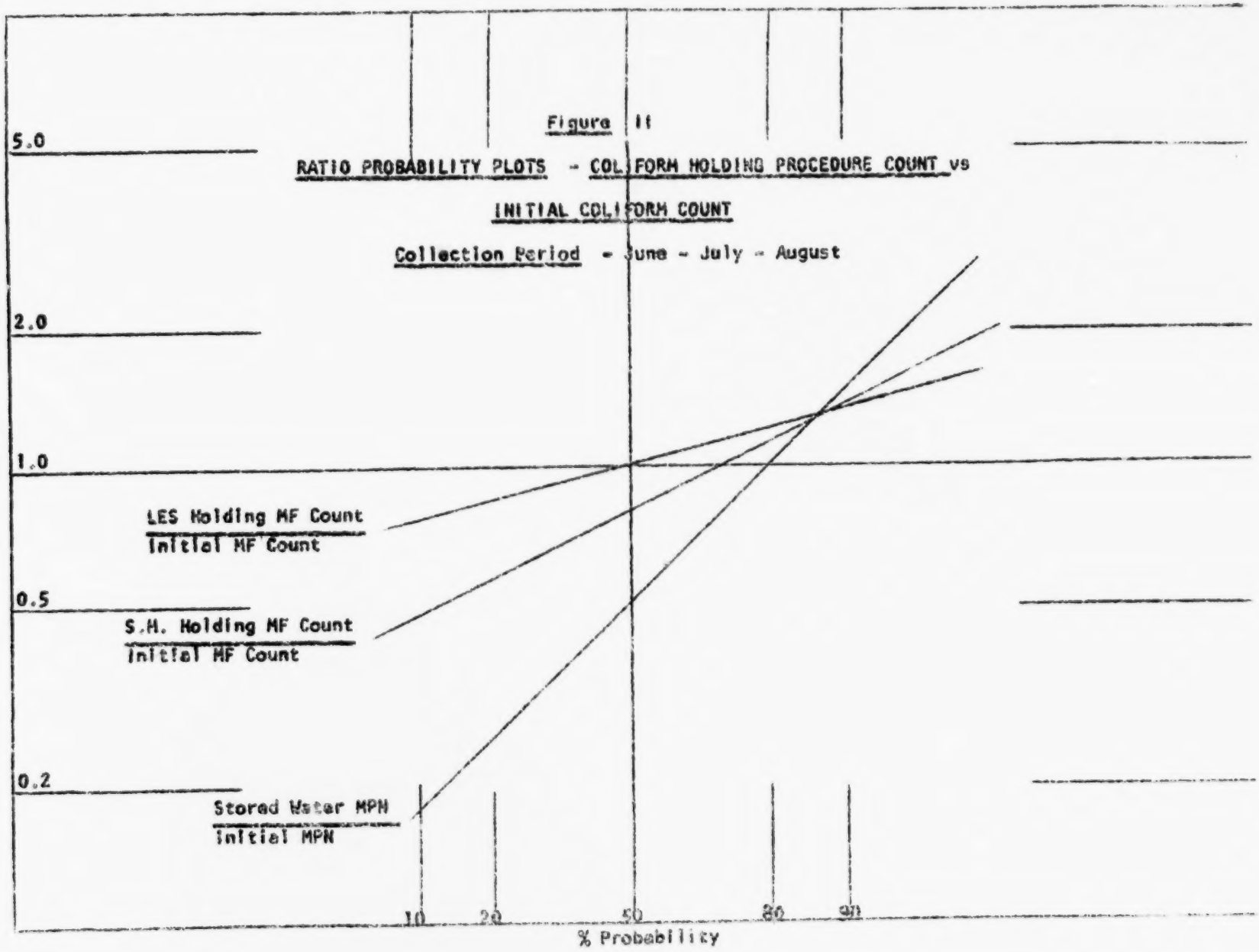


Figure 1 RATIO PROBABILITY PLOTS - COLIFORM HOLDING PROCEDURE COUNT vs
INITIAL MF COLIFORM COUNT
Collection Period - December - January - February
Stored Water MF Count / Initial MF Count
LES Holding MF Count / Initial MF Count
S. M. Holding MF Count / Initial MF Count
5.0
2.0
1.0
0.5
0.2
10
20
50
80
90
Probability

Figure 11

RATIO PROBABILITY PLOTS - COLIFORM HOLDING PROCEDURE COUNT vs INITIAL COLIFORM COUNT

Collection Period - June - July - August



From an analysis of these tables, the following conclusions can be made concerning the various Holding Procedures:

(1) Standard Methods Holding Technic - In cold weather, fifty percent of the analyses gave a result that is at least 40% below the original coliform density of the sample, while seventy-five percent of the analyses gave a result that is at least 20% below the original coliform density. During the summer months, this procedure yields a higher coliform recovery, but still fifty percent of the analyses gave a result that is at least 19% below the original coliform density of the sample and twenty-five percent of the analyses gave a result that was at least 38% below the original coliform count.

(2) L.E.S. Holding Technic - This procedure, for the fifty samples analyzed during both the cold and warm weather months, gave median ratios of 1.06 and 1.02 respectively. The 25-75 percentile ranges of this technic represent fairly normal dispersion expected from replicate sampling. The cold weather data appear to indicate that the L.E.S. Holding Technic recovers a greater number of coliforms than the initial MF procedure. This tendency may be connected with the long resuscitation period presented to the weak coliform strains by the L.E.S. Holding Medium coupled with the moderate temperatures encountered during the holding period.

(3) Water Holding Environment - The ratios of the stored water MF counts to the initial MF count for the cold weather sampling period indicate a great fluctuation of the coliform densities on storage. While the median ratio is 0.71, the 25-75 percentile range, namely 0.25-1.40, indicates the wide fluctuation that may occur in a water sample during transport. The membrane

filter method, for measuring coliform densities in water, was found extremely difficult to use on stored samples during the warm weather period of this study. High non-coliform counts on the membrane surface made the counting of sheen colonies difficult and many times impossible. For the warm weather analyses, the MPN data are presented in Table II and Figure II. These data indicate that fifty percent of the analyses performed on the stored water sample would yield a result that is at least 49% below the original coliform count of the sample. Judging from the MF data presented in Table II it would appear that an analysis made on a sample collected and shipped during the winter months would yield a closer estimate of the original density than a sample collected, shipped and analyzed during the summer months.

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As a result of this research grant on various Holding Procedures for coliform bacteria, the USPHS, Basic Data Water Quality Section is presently designing a year's study program to evaluate the potential of the L.E.S. Holding Procedure.

Reference

1. McCarthy, J. A. Storage of Water Samples for Bacteriological Examinations A. J. P. H. 47:971 (1957)

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Section IV - Rapid MPN Procedure for the Estimation of E. coli Type I in Shellfish, Benthal Deposits and Turbid Waters

Introduction

In the field of Shellfish Control, at the present time there appears to be a desire to develop, and to apply, a more significant bacteriologic indicator for pollution assessment than the coliform group. This emphasis on methodology has arisen because of (1) the growing acceptance that the separation of the coliform group into fecal and non-fecal subdivision is justified and reasonable, based on the hypothesis that coliform bacteria of fecal origin represent greater danger than those native to other environments, (2) the frequent, perplexing inability to relate the coliform densities found in certain shellfish areas to the potential public hazard as judged from a sanitary survey and (3) the mounting incrimination of shellfish as a vehicle for the transmission of viral disease, specifically infectious hepatitis.

The coliform group has been the major parameter for assessing the bacterial quality of shellfish but its predominating position is being challenged by two subdivisions of the parent group, namely the "so-called fecal coliform" group and E. coli type I specie. Many bacteriologists tend to favor these indicators as a more positive and meaningful basis of estimating pollution than the total coliform group since their presence is generally regarded as positive proof of fecal contamination, whereas the recovery of the coliform group is considered a less certain indication of the presence of fecal pollution.

Various investigators have shown that 90 to 100% of the coliform organisms issuing from the intestine of warm blooded animals are E. coli

type I. It is generally maintained that E. coli is found only in waters which are liable to a direct or indirect contamination by matter originating in the animal or human intestine. E. coli type I therefore represents an ideal indicator of fecal contamination.

The density of E. coli type I in a shellfish sample can be obtained by the present lactose MPN procedure or by the E. C. MPN procedure but involved a great deal of laboratory manipulation and consumes an excessive period of time for this approach to be an acceptable method for control purposes. The routine analysis for this organism in shellfish and sea water has awaited the development of an accurate, simple procedure which does not involve the time consuming steps of purification and differentiation.

Our study on the "Enumeration of E. coli I by the Membrane Filter Technic" (Cf. Section II of this Report) showed that the biochemical test for indole production at an elevated temperature could be exploited as a selective test for this coliform specie. It was reported that practically all E. coli I organisms are able to perform this reaction while only a few other bacterial species can produce indole at an elevated temperature.

All quantitative methods for E. coli I based on lactose fermentation at an elevated temperature inherently possess low specificity, because varying percentages of the remaining coliform species, principally -+-- and --++ are also able to ferment lactose at an elevated temperature. These species obviously will not by themselves interfere with the specificity of a test based on indole production. The production of indole at an elevated temperature also

appears to be a basis for a more inclusive test for E. coli I since it demonstrates the sizeable percentage of E. coli I organisms which are non-lactose fermenting or ferment lactose only after a prolonged incubation period. Ewing [1] reports that such anaerogenic E. coli organisms comprise 5.5% of this organism's density in human feces. In the 7th edition of Bergey's Manual these organisms are relegated to the genus Paracolobactrum in which totally different microorganisms are grouped together simply because of their reluctance to utilize lactose. This Paracolobactrum genus is not recognized by the International Enterobacteriaceae Subcommittee. Even a neophyte in bacteriological science can appreciate the soundness of the practice to classify microorganisms by the overall spectrum of their biochemical reactions and not by a single ability. Thus, the so-called anaerogenic E. coli I, previously Paracolobactrum coliforme, although deficient in the lactose enzyme, is proficient in most essential traits of the classical E. coli.

Because of interference by turbidity and colloidal matter, the TBA Membrane Filter method for E. coli I obviously cannot be used for all samples that are submitted for analysis to a Sanitary Bacteriological Laboratory. Muds, macerated shellfish, milk and highly turbid waters with low E. coli densities fall into this category.

After considerable preliminary evaluations on medical constituents, growth patterns, transfer methods and time studies (Cf. Progress Report WP00202-04) an analytical procedure was tentatively programmed to include the following steps:

(1) The macerated sample would be inoculated into an MPN array

of tubes of lauryl tryptose broth containing a glass rod that had been roughened on a grinding wheel at its immersed end.

(2) The tubes would then be placed in a 35°C incubator overnight to permit growth of the bacterial flora with niduses (nests) of bacteria forming on the immersed surface of the glass rod.

(3) At the start of the following work day, the glass rods would be removed from the growth tubes and transferred to a small volume selective confirmatory broth . (Cf Progress Report for basis of formulation) All positive tubes were inoculated into tubes of E. C. Medium and incubated in a water bath at 44.5°C for 24 hours.

(4) The confirmatory tubes were then placed in a water bath regulated at $44.5^{\circ} \pm 0.2^{\circ}C$ for six hours.

It was theorized that the E. coli Type I colonies transferred by the glass rod would be present in sufficient numbers in the small volume of confirmed broth to cleave sufficient molecules of tryptophane to yield a color reaction with a chemical indicator of p-Dimethyl amino benzaldehyde.

(5) The positive and negative tube pattern that developed in the MPN array would yield the MPN code of the most probable number of E. coli Type I in the macerated sample.

In order to determine the inclusiveness and selectivity of this procedure, the glass rod removed from each positive indol tube was used as a streaking tool on E. M. B. agar and each positive E. C. tube was streaked on E. M. B. and the coliform growth, when present, was differentiated. The latter procedure served as the control for the comparative recovery study.

Fifty shellfish samples that were subjected to the two selective procedures yielded the results presented in Table I. The results of the LES E. coli I procedure were obtained in one day while the finalized E. coli I results originating from the E. C. procedure were not reportable until nine days after the initial planting of the sample. The data indicate good to excellent correlation between the results obtained by the two independent methods. It was observed that the LES indol testing method produced low results in those samples possessing initially low E. coli I densities. (Refer to Table I samples) This occurrence is generally believed to be caused by the transfer of insufficient E. coli I organisms from the growth medium to the confirmation medium. It is theorized that the inoculated sample, in these cases, contain one to several E. coli, at most, and large numbers of other bacterial species. During the growth phase the few E. coli organisms compete with the many bacterial species for food and more important for growth sites on the glass rod. At the conclusion of the growth phase, the microbial organisms attached to the glass rod are predominantly non E. coli I and thus the necessary inoculum of E. coli I needed from an enzymatic kinetic standpoint to produce a positive indol test in six hours is not achieved.

Table I

Sample No.	E. coli I MPN LES Rapid Method (1)	E. coli I MPN EC Tube Differentiation (2)
1	490	490
2	1300	1700
3	5400	5400
4	3500	1100
5	640	460
6	340	1300
7	52	170
8	68	490
9	1700	1700
10	3500	2400
11	2400	2400
12	1700	1300
13	16,000	16,000
14	1700	1700
15	1100	1100
16	9200	5400
17	16,000	16,000
18	790	790
19	700	700
20	2400	2400
21	9200	9200
22	700	490
23	1700	1700
24	5400	5400
25	3500	3500
26	330	330
27	490	490
28	2200	2200
29	790	790
30	68	110
31	45	130
32	3500	5400
33	9200	9200
34	1800	1800
35	1100	1100
36	3500	3500
37	950	950
38	5400	5400
39	5400	5400
40	3500	3500
41	110	170
42	700	700
43	490	490
44	230	230
45	1300	1300
46	1300	1300
47	270	790
48	68	170
49	120	210
50	20	20

During this evaluation of the rapid method for E. coli Type I, sporadic weak tests for indole were observed at the end of the confirmation period, from which it was impossible to isolate E. coli Type I. After considerable investigation, it was determined that the peptone, tryptose present in the lauryl tryptose broth formula, contained 0.64% tryptophane and was the direct cause of the false weak positive indole tests. It was theorized that non-E. coli bacteria attacked the tryptophane in the growth broth at 35°C and produced sufficient indole to permit the glass rod to carry over an amount necessary to yield a weak indole test in the confirmatory procedure.

To combat this interferance with the selectivity of the proposed test, a new growth medium was formulated. This presumptive medium was basically lactose broth to which was added the amounts of buffer, sodium chloride and lauryl sulfate contained in lauryl sulfate broth. Thus, the Modified Lactose Broth formula is as follows:

Lactose	5 grams
Peptone	5 grams
Beef Extract	3 grams
Potassium MonoPhosphate	2.75 grams
Potassium DiPhosphate	2.75 grams
Sodium Chloride	5 grams
Sodium Lauryl Sulfate	0.1 grams
Water	1 liter

The employment of this formula as the growth broth eliminated the appearance of false indole producers at the conclusion of the confirmed test.

To increase the inclusiveness of the proposed procedure, it was decided to eliminate the oxgall from the preliminary confirmatory media and add a small increment (0.1 gram/liter) of tryptophane. The final confirmatory broth possessed the following composition:

Tryptone	10 grams
Potassium Monophosphate	2 grams
Potassium Diphosphate	2 grams
Tryptophane	0.1 gram
Distilled Water	1 liter

The employment of this confirmatory medium in conjunction with the modified lactose broth as the growth medium produced a very satisfactory procedure embodying satisfactory inclusiveness and selectiveness for E. coli Type I in shellfish, benthal deposits and turbid waters.

A. Summary of Apparatus, Media & Procedure

1. Laboratory Apparatus

(a) Water Bath - regulated at 44.5°C with a tolerance of ± 0.2°C when filled to a depth of 2 1/2 inches. A bath equipped with a recirculation pump is preferred.

(b) Glass Rods - 3 x 150 mm glass rods with approximately 30-40 mm at one end roughed by a medium coarse grinding wheel.

(c) Test tubes - sizes 10 x 75 and 15 x 150 mm.

(d) Test tube racks - test tube support racks similar to the U. S. Army Medical - 20 place, 16 mm diameter holes are ideal for this purpose.

2. Media and Reagent

(a) Modified Lactose Broth - To each liter of distilled water add 23.6 gms. of modified Lactose Broth. Distribute 10 ml portions in 15 x 150 mm test tubes, insert glass rod, roughed and immersed in the broth and plug with cotton. Autoclave at 121°C for 15 minutes.

(b) Confirmatory Broth - Alternate Distribution Procedures

(1) Distribute 1 ml portion in 10 x 75 mm tubes, nest in glass beakers (250 ml), cap beakers with kraft paper and sterilize in autoclave at 121°C for 15 minutes. Note: cotton plugging of individual tubes is not necessary. Store sterilized media in refrigerator.

(2) Distribute 100 ml portions in erlenmeyer flasks, plug and autoclave at 121°C for 15 minutes. Store in refrigerator. As needed, pipette 1 ml portions into 10 x 75 mm tubes that have been sterilized by either dry or wet methods.

If tubes are to be used within a few days after preparation, it is recommended that the first procedure be adopted.

(c) p-Dimethylaminobenzaldehyde reagent (p-DAB). Dissolve 5 grams of p-DAB in 75 ml of iso-amyl alcohol and 75 ml concentrated hydrochloric acid. If a large scale testing program is planned, an automatic burette simplifies the dispensing of this reagent. Store in refrigerator.

3. Detailed Procedure

The following are the four steps in the procedure:

(a) Inoculate replicate modified Lactose broth tubes with appropriate serial dilutions of the sample to be tested. Incubate at 35°C overnight.

(b) Next morning, at the beginning of the workday, remove the rods from the growth tubes and insert them into the confirmatory broth. Incubate in water bath regulated at 44.5°C ± 0.3°C. No plugging of tubes is necessary.

(c) After six hours incubation, the confirmatory tubes are tested for the presence of Indole by adding 0.3 ml of p-DAB reagent. If verification of the Indole tubes is desired, the glass rod is removed prior to the addition of the p-DAB reagent to allow subculturing.

(d) The MPN code obtained from the positive and negative tube pattern obtained in step #3 is translated into an MPN by means of an MPN Table.

B. Laboratory Evaluation of Method

The application of the above procedure to estimate the E. coli type I densities in shellfish exhibited the results that are presented in Table II, Column II. In Column III, the E. coli I MPN's obtained from the E. C. Test followed by differentiation of the bacterial specie found in each positive tube are presented. These shellfish samples were collected during the winter, spring and summer months and therefore represent a cross-section of the types of flora present in shellfish during the seasons of the year.

TABLE II

Sample No.	E. coli Type I LES Rapid Method	E. coli Type I by E. C. Tube Differentiation
1	490	490
2	230	230
3	700	700
4	3500	3500
5	220	490
6	130	230
7	230	230
8	490	490
9	1100	1100
10	2400	2400
11	790	790
12	1300	1300
13	3500	3500
14	3500	3500
15	460	460
16	330	330
17	700	700
18	460	460
19	330	330
20	330	330
21	1700	1700
22	2400	2400
23	330	330
24	20	45
25	490	490
26	790	790
27	1700	1700
28	700	700
29	170	170
30	330	330

Twenty-seven out of the thirty samples analyzed gave the same result by the two procedures. In only three of the samples did the Rapid LES E. coli I Method fail to indicate a lower MPN than the control E. C. plus differentiation Method. It is important to keep in mind that the LES Method required only one day to obtain its result while the control method required nine days after the initial planting of the sample to obtain its result.

Summary

The procedure developed for E. coli type I in Shellfish, Benthal deposits and turbid waters possesses the qualities of rapidity, satisfactory inclusiveness and high selectivity, which are desirable in a standard bacteriological procedure. The method is based on E. coli type I's ability to produce indole while exposed to an incubation temperature of 44.5°C and the inability of other bacterial species common to water, sewage and shellfish to perform this biochemical reaction.

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